

enclose herewith a Second Substitute Sequence Listing in paper and computer readable form.


Applicants respectfully request reconsideration of the instant application in view of the following amendments and remarks.

IN THE SPECIFICATION

Please **amend** the paragraphs beginning at page 6, line 20 and ending at page 6, line 32 with the following rewritten paragraphs:

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The instant invention provides immunogenic peptides capable of eliciting protective immunity against botulinum neurotoxin of serotypes A-G.

 The instant invention also provides vaccines capable of eliciting protective immunity against botulinum neurotoxin, where the vaccines do not act as neurotoxins themselves.

The instant invention further provides methods for preparing non-toxic peptides for use in vaccines against botulinum neurotoxin by growing recombinant organisms which express the peptides.

The instant invention also provides methods for fast and efficient purification of the non-toxic peptides from cultures of recombinant organisms.

C<sup>1</sup> These and other aspects are illustrated by one or more of the following embodiments of the present invention.

Please **amend** the paragraphs beginning at page 9, line 20 and ending at page 11, line 12 with the following rewritten paragraphs:

C<sup>2</sup> Figures 1A and 1B respectively show the nucleotide sequence and the encoded amino acid sequence for a synthetic gene encoding the H<sub>c</sub> fragment of BoNT serotype A (SEQ ID NOS:1 and 2).

Figures 2A and 2B respectively show the nucleotide sequence and the encoded amino acid sequence for a synthetic gene encoding the H<sub>c</sub> fragment of BoNT serotype A (SEQ ID NOS:3 and 4).

Figures 3A and 3B respectively show the nucleotide sequence and the encoded amino acid sequence for a synthetic gene encoding the H<sub>c</sub> fragment of BoNT serotype A (SEQ ID NOS:5 and 6).

Figures 4A and 4B respectively show the nucleotide sequence and the encoded amino acid sequence for a synthetic gene encoding the H<sub>c</sub> fragment of BoNT serotype B (SEQ ID NOS:7 and 8).

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Figures 5A and 5B respectively show the nucleotide sequence and the encoded amino acid sequence for a synthetic gene encoding the H<sub>c</sub> fragment of BoNT serotype C (SEQ ID NOS:9 and 10).

Figures 6A and 6B respectively show the nucleotide sequence and the encoded amino acid sequence for a synthetic gene encoding the H<sub>c</sub> fragment of BoNT serotype D (SEQ ID NOS:11 and 12).

62  
Figures 7A and 7B respectively show the nucleotide sequence and the encoded amino acid sequence for a synthetic gene encoding the H<sub>c</sub> fragment of BoNT serotype E (SEQ ID NOS:13 and 14).

Figure 8 shows the nucleotide sequence for a synthetic gene encoding the H<sub>c</sub> fragment of BoNT serotype E and the encoded amino acid sequence (SEQ ID NOS:35 and 36).

Figures 9A and 9B respectively show the nucleotide sequence and the encoded amino acid sequence for a synthetic gene encoding the H<sub>c</sub> fragment of BoNT serotype F (SEQ ID NOS:15 and 16).

Figures 10A and 10B respectively show the nucleotide sequence and the encoded amino acid sequence for

a synthetic gene encoding the H<sub>C</sub> fragment of BoNT serotype G  
(SEQ ID NOS:17 and 18).

Figures 11A and 11B respectively show the  
nucleotide sequence and the encoded amino acid sequence for  
a synthetic gene encoding the H<sub>N</sub> fragment of BoNT serotype A  
(SEQ ID NOS:19 and 20).

Figures 12A and 12B respectively show the  
nucleotide sequence and the encoded amino acid sequence for  
a synthetic gene encoding the H<sub>N</sub> fragment of BoNT serotype B  
(SEQ ID NOS:21 and 22).

Figures 13A and 13B respectively show the  
nucleotide sequence and the encoded amino acid sequence for  
a synthetic gene encoding the H<sub>N</sub> fragment of BoNT serotype C  
(SEQ ID NOS:23 and 24).

Figures 14A and 14B respectively show the  
nucleotide sequence and the encoded amino acid sequence for  
a synthetic gene encoding the H<sub>N</sub> fragment of BoNT serotype D  
(SEQ ID NOS:25 and 26).

Figures 15A and 15B respectively show the  
nucleotide sequence and the encoded amino acid sequence for  
a synthetic gene encoding the H<sub>N</sub> fragment of BoNT serotype E  
(SEQ ID NOS:27 and 28).

Figures 16A and 16B respectively show the nucleotide sequence and the encoded amino acid sequence for a synthetic gene encoding the H<sub>N</sub> fragment of BoNT serotype F (SEQ ID NOS:29 and 30).

Figures 17A and 17B respectively show the nucleotide sequence and the encoded amino acid sequence for a synthetic gene encoding the H<sub>N</sub> fragment of BoNT serotype G (SEQ ID NOS:31 and 32).

Figures 18A and 18B respectively show the nucleotide sequence and the encoded amino acid sequence for a synthetic gene encoding the H<sub>C</sub> fragment of BoNT serotype F (SEQ ID NOS:33 and 34).

Figures 19A, 19B, and 19C. Figure 19A shows the AT base content of a putative fragment C region in native *C. botulinum* DNA. Figure 19B shows the reduced AT content after the first design (rBoNTF(Hc)1) of the synthetic gene. Figure 19C shows the AT content of the final gene design (rBoNTF(Hc)2) used to express recombinant rBoNTF(Hc) in *P. pastoris*.

Figures 20A and 20B. Figure 20A shows an SDS-PAGE gel and Figure 20B shows a Western blot of samples at various steps along the rBoNTF(Hc) purification. Lanes

from both figures are identical except lane 1, where SDS-PAGE shows Novex mark 12 wide-range molecular weight markers and Western blot shows Novex See Blue prestained molecular weight markers. Lane 2 is the cell lysate, lane 3 is the cell extract, lane 4 is the cell extract after dialysis, lane 5 is pool of rBoNTF(Hc) positive fractions after Mono S column chromatography, and lane 6 is pool of rBoNTF(Hc)-positive fractions after hydrophobic interaction chromatography.

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Figures 21A and 21B show purification of rBoNTF(Hc) by sequential chromatography. Figure 21A shows Mono S cation exchange chromatography of extract from *P. pastoris*. Proteins were eluted with increasing NaCl gradient. Fractions positive for rBoNTF(Hc) by Western analysis were pooled individually and subjected to hydrophobic interaction chromatography (the results of which are shown in Figure 21B) and proteins were eluted with a decreasing ammonium sulfate gradient. In both panels, protein monitored by A280nm is recorded on the left axis and elution conditions are recorded on the right axis, with the gradient trace laid over the chromatogram.

Please **amend** the paragraph beginning at page 12, line 7 and ending at page 12, line 14 with the following rewritten paragraph:

*C3* (Kozaki) et al. (in "Antibodies against Botulism Neurotoxin", L.L. Simpson, ed., 1989, Academic Press, New York) suggested that a protective epitope might be present in the 50 kDa carboxyl terminus (HC) region of the protein. Thompson et al. (1990, *Eur. J. Biochem.* **189**:73-81) deduced the amino acid sequence for the serotype A botulinum toxin. *Type A* (DasGupta et al. (1990, *Biochemie*, **72**:661-664) identified the "nick" site for post-translational cleavage of the expressed toxin polypeptide, from which the sequence of the heavy chain can be deduced as SEQ ID NO:41. See also Krieglstein, et al., 1994, *J. Protein Chem.*, **13**:49-57.

Please **delete** the sequence paragraph beginning at page 12, line 15 and ending at page 12, line 31.

Please **amend** the paragraph beginning at page 13, line 1 and ending at page 13, line 6 with the following rewritten paragraph:

*CH* Whelan et al. (*Appl. Environ. Microbiol.* **58**:2345-2354, 1992) have deduced the amino acid sequence for the

serotype B botulinum toxin. Schmidt, et al. (1985, Arch. Biochem. Biophys., 238:544-548) provided N-terminal sequence information for the heavy chain resulting from post-translational cleavage of the expressed toxin polypeptide, and the sequence of the heavy chain can be deduced from this information as SEQ ID NO:42.

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Please **delete** the sequence paragraph beginning at page 13, line 8 and ending at page 13, line 24.

Please **amend** the paragraph beginning at page 36, line 17 and ending at page 36, line 17 with the following rewritten paragraph:

The sequence of the C fragment of the A chain was deduced as SEQ ID NO:38.


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Please **delete** the sequence paragraph beginning at page 36, line 18 and ending at page 36, line 26.

Please **amend** the paragraph beginning at page 36, line 32 and ending at page 36, line 32 with the following rewritten paragraph:




The sequence for the synthetic gene is SEQ ID

 NO:37.

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Please **delete** the sequence paragraph beginning at page 37, line 1 and ending at page 37, line 27.

Please **amend** the paragraph beginning at page 38, line 6 and ending at page 38, line 7 with the following rewritten paragraph:

 The C fragment for botulism toxin serotype B of Whelan was studied and the portion of the protein having the sequence of SEQ ID NO:40 was defined as the C fragment.

Please **delete** the sequence paragraph beginning at page 38, line 8 and ending at page 38, line 16.

Please **delete** the paragraph beginning at page 38, line 17 and ending at page 38, line 17.

Please **amend** the paragraph beginning at page 38, line 18 and ending at page 38, line 25 with the following rewritten paragraph:

CO The synthetic gene for expression in *E. coli* was produced in the manner described for synthesis of the gene for the C fragment of the A strand, namely, using a large number of oligomers of approximately 60-65 bases corresponding to the sequences of the + and - strands with overlaps of 7 bases. The oligomers were allowed to anneal and were ligated to form subunits of 250-300 base pairs each. Each subunit had been designed to have restriction sites at their termini which allowed them to be assembled in the right order to form the complete gene. The synthetic gene encoding the C fragment of the B toxin is SEQ ID NO:39.

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Please **delete** the sequence paragraph beginning at page 38, line 26 and ending at page 39, line 21.

IN THE SEQUENCE LISTING

Please **delete** the Sequence Listing presently of record and substitute, therefor, the attached Second Substitute Sequence Listing.